



## **Oxidative Stress Markers in Children with Autism Spectrum Disorders**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors.  
Author MEGF was the principal investigator, designed the study, conducted the data analysis and wrote of the paper. Author M-LDH oversaw the laboratory measurements and performed the statistical analysis. Authors HV, CM and RG were the study neurologists, oversaw patient care and assisted with interpreting the results, Author EN classify the participants for the group control. Authors LB and MR assisted with interpreting the results. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** The etiology of autism spectrum disorders (ASD) remains elusive, but oxidative stress has been suggested to play a pathological role. The understanding of the potential role of oxidative stress in the etiopathogenesis of autism would be very useful for earlier clinical, therapeutic or preventive strategies.

**Sample:** To evaluate the redox status, we quantified the activity of the antioxidant enzyme catalase (CAT), glutathione concentration (GSH) and markers of damage to biomolecules, *malonyldialdehyde* (MDA) and 8-hydroxy-2deoxyguanosine (8OHdG) in peripheral blood samples.

**Place and Duration of Study:** Sample: Department of Neuropediatrics and Technology Science Division. International Center for Neurological Restoration (CIREN), Havana, Cuba. May 2011- June 2012.

**Methodology:** We included 45 children with autism (36 males and 9 females, age-

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range from 3 to 11 years). 42 children of the same age were selected as a control group. The diagnosis of autism was made based on the criteria of autistic disorders as defined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV) (American Psychiatric Association, 1994).

**Results:** The total GSH content in autistic patients was significantly lower compared with the control group ( $0.24 \pm 0.162$  vs.  $0.94 \pm 0.115$ , respectively,  $p \leq 0.001$ ). Higher serum CAT, MDA and 8OHdG levels were found in children with autism compared with controls (CAT,  $2.836 \pm 0.479$  vs.  $0.689 \pm 0.157$ ,  $p \leq 0.001$ ; MDA  $8.6 \pm 0.5$  vs.  $1.76 \pm 0.33$   $p \leq 0.001$ , and 8OHdG  $13.134 \pm 1.33$  vs.  $1.46 \pm 0.326$ ,  $p \leq 0.001$ ).

**Conclusion:** The present study supports the notion that oxidative stress is associated with autism, but additional researches are needed to investigate how it may contribute to autistic pathophysiology and these studies are currently in progress.

*Keywords: Autism; catalase; glutathione; malonyldialdehyde; oxidative stress and 8-hydroxy-2-deoxyguanosine.*

## 1. INTRODUCTION

Free radicals are molecules with unpaired electrons which are known to have strong oxidant effects. The reduction of one electron of oxygen ( $O_2$ ) produces the superoxide anion radical ( $O_2^-$ ), which is converted rapidly to  $O_2$  and hydrogen peroxide ( $H_2O_2$ ) by the enzyme superoxide dismutase (SOD).  $H_2O_2$  is reduced to water by the enzyme catalase (CAT) and glutathione peroxidase (GPx). In certain conditions,  $H_2O_2$  persists, and it is involved in reactions with transition metal-ion to produce hydroxyl radicals ( $\cdot OH$ ) which are highly toxic and provoke an imbalance in the cellular redox state towards the pro-oxidant status [1].

Excessive production of free radicals or impaired antioxidant mechanism may cause oxidative stress which may induce several pathophysiological processes. The two main role of cellular antioxidant defense mechanism are to prevent the generation of free radicals and to inactivate them after generation. Impaired antioxidant defense mechanism can result in cell membrane damage, alteration in membrane fluidity and permeability and oxidative changes in proteins, lipids and DNA [2].

The nervous tissue is especially vulnerable to the oxidant threat because of its high rate of aerobic metabolism and elevated concentrations of iron and readily oxidizable molecules like catecholamines and polyunsaturated fatty acids [3]. In addition, several proteins with relevant roles in normal brain function are targets for oxidative modifications that modulate their activity. ROS play a crucial role in both normal and pathological cell signaling process such as calcium homeostasis, kinase activity and gene regulation. Oxidative stress controls the activities of receptor and nonreceptor types of protein tyrosine kinases, protein kinase C and transcription factors such as NF $\kappa$ B [4]. In this context, reactive oxygen species are considered to act not only as deleterious agent but also cellular messengers.

Autism Spectrum Disorders (ASD) is characterized by impaired social interaction, problems with verbal and nonverbal communication, and repetitive or severely restricted activities and interest. Limited scientific advances have been made regarding the causes of ASD, with general agreement that autism could result from the interaction between genetic and environmental factors with oxidative stress as a potential link between them [5].

Several environmental toxins, such as heavy metals, including mercury and pesticides have been implicated in autism [6]. These factors in turn share the ability to increase oxidative stress. It is possible that the autistic pathology could result from exposure to high levels of environmental pro-oxidants and a genetic predisposition to oxidative stress triggers.

With respect to autism, the notion of enhanced oxidative stress has been derived from several lines of evidences: elevated nitric oxide concentration [7,8] thiobarbituric acid reactive substance levels and xantine oxidase activity [9] have been detected in the red blood cells of autistic individuals. Consistent with these increased oxidative stress biomarkers in children with autism, a reduced endogenous antioxidant capacity, specifically the total GSH levels, altered GPx, SOD and CAT activities were found in autistic individuals compared to controls [10].

Additional indications of oxidative stress in autism are derived from evidence of impaired energy metabolism. Reduced synthesis of adenosine triphosphate (ATP) and higher lactate and pyruvate levels may suggest mitochondrial dysfunction in autism [11]. The most important function of mitochondria is producing ATP, the primary energy currency in the brain and in the body. Increased reactive oxygen species (ROS) metabolism induced by dysfunctional mitochondria could elicit chronic oxidative stress.

In this study we determined serum levels of antioxidants (CAT) enzymatic activity and GSH concentration, malondialdehyde (MDA), a lipid peroxidation indicator, and the biomarker of DNA hydroxylation, 8-hydroxy-2deoxyguanosine (8OHdG), as a marker for oxidative damage to DNA, in autistic patients and age-matching control children.

## **2. MATERIALS AND METHODS**

### **2.1 Subjects**

A traverse study one carries out in forty five children with autism (36 males and 9 females, age-range from 3 to 11 years, Mean  $\pm$  SD: 7.66  $\pm$  2.68). Forty two children of the same age were selected as a control group (30 males and 12 females, age range from 3 to 11 years, Mean  $\pm$  SD: 7.11  $\pm$  2.62).

The diagnosis of autism was made based on the criteria of autistic disorders as defined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM IV) (American Psychiatric Association, 1994) [12].

All children with a history of seizure disorders, severe head injury, psychotic disorder and any other major acute or chronic physical or mental illnesses were excluded from the control group. All patients were in good physical health and subjects with serious systemic illnesses were excluded.

Children who had been taking vitamin or mineral supplements during the 2-month prior to the study were also excluded.

## **2.2 Ethics Approval and Consent**

A written consent was obtained from the parents of each individual case, according to the guidelines of the Ethics Committee (Registration: 5/2012, Record No. 3) of the International Center for Neurological Restoration (CIREN), Havana, Cuba.

## **2.3 Blood Samples**

After asepsis, five milliliters of blood were extracted by venous puncture from all subjects. The blood was allowed to clot and serum was extracted and stored at -70 °C until use. The storage time was less than one month.

## **2.4 Chemicals**

All chemicals used in this study were of analytical grade. Malonaldehyde bis (dimethylacetal), 99% was a product from Sigma (USA). GSH was purchased from BDH, Analar (Poole, UK) and the 8OHdG ELISA kit was a product of the JaiCA, Japan.

## **2.5 Biochemical Analyses**

### **2.5.1 Determination of the CAT enzymatic activity**

CAT is the primary enzyme involved in direct elimination of H<sub>2</sub>O<sub>2</sub>. The activity of this enzyme was determined according to Aebi [13]. The capacity of CAT to transform H<sub>2</sub>O<sub>2</sub> was measured by monitoring the decomposition of H<sub>2</sub>O<sub>2</sub> at 240 nm in 0.06M sodium phosphate buffer, pH 7 at 37 °C. One unit of enzymatic activity was considered as a quantity of enzyme necessary to transform 1 μmol of H<sub>2</sub>O<sub>2</sub> in 1 min at 37 °C. The molar extinction coefficient of 0.0436 (mmol L<sup>-1</sup>)<sup>-1</sup> cm<sup>-1</sup> was used.

### **2.5.2 Determination of the MDA concentration [14]**

MDA is an end product of peroxidation of polyunsaturated fatty acids and related esters and is used as marker of lipid peroxidation. This technique is based on the capacity of reaction of TBA with MDA to form an adduct that absorbs light at 535 nm. A standard curve of MDA was used to estimate the concentration values in the sample.

### **2.5.3 Determination of the GSH concentration**

GSH is the most important endogenous antioxidant for detoxification and elimination of environmental toxins and ROS. Total GSH was determined by Tietze's enzymatic recycling assay (Azbill et al.) [15]. The serum was incubated for 25 min at 37°C in a medium containing 0.24 mM NADPH, 6.3 mM EDTA, 0.67 mM DTNB and 143 mM sodium phosphate pH 7.5. After the addition of 1U/ml GRD, the absorbance increase at 412 nm due to the formation of 5-thio-nitrobenzoate was recorded. The concentration values were calculated from a GSH curve.

### **2.5.4 Determination of 8-hydroxy-2-deoxyguanosine, (8-OHdG) concentration [16]**

8-OHdG is a product of oxidatively damaged DNA formed by hydroxyl radicals, singlet oxygen and direct photodynamic action. 8-OHdG test is a competitive enzyme-linked

immunosorbent assay (ELISA kit, JaICA, Japan) utilizing a monoclonal antibody (clone N45.1), which is highly specific for 8-OHdG.

### 2.6 Statistical Analysis

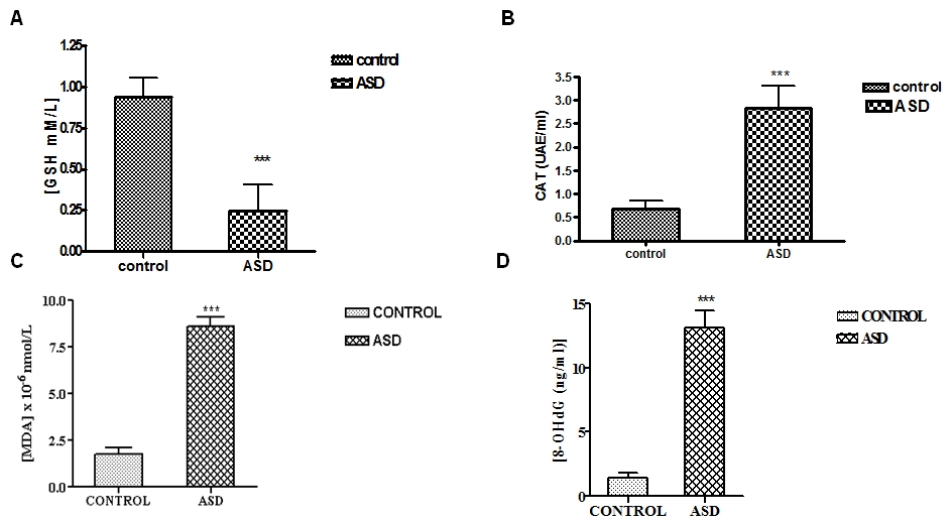
The values are expressed as mean ± S.D. Normal distribution and homogeneity of variance of the data were tested by the Kolmogorov – Smirnov and Levene test, respectively. For those variables that did not meet these premises (CAT enzymatic activity and GSH concentration), the Mann–Whitney U nonparametric test was used for the comparison between patients and control individuals. The MDA and 8-OHdG concentrations were analyzed by the independent t-test. A p value lower than 0.05 was considered significant.

### 3. RESULTS AND DISCUSSION

Results are presented as mean ± SD for each measured parameters in the Table 1. Figures of each parameter in 45 autistic patients and 42 controls are also shown.

**Table 1. Oxidative stress indicators in serum of autistic and control children**

Oxidative stress indicators	Group	N	Mean ± SD	p value
GSH Concentration (mM/L)	Autistic	45	0.24 ± 0.162	p ≤ 0.001
	Control	42	0.94 ± 0.115	
CAT antioxidant enzyme (UAE/L)	Autistic	45	2.836 ± 0.479	p ≤ 0.001
	Control	42	0.689 ± 0.157	
MDA (nmol/L)	Autistic	45	8.6 ± 0.5	p ≤ 0.001
	Control	42	1.76 ± 0.33	
8-OHdG (ng/ml)	Autistic	45	13.134 ± 1.33	p ≤ 0.001
	Control	42	1.46 ± 0.326	



**Fig. 1. Oxidative stress markers in 45 ASD patients and 42 age-matching control children**

Antioxidants markers A: glutathione concentration (GSH) and B: antioxidant enzyme catalase (CAT). Indicators of oxidative damage, C: malonyldialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8OHdG). \*\*\* (p<0.001)

### **3.1 Discussion**

GSH plays an important role in a several cellular process including cell differentiation, proliferation, and apoptosis. Therefore, disturbances in GSH homeostasis are implicated in the etiology and/or progression of a number of human diseases [17].

GSH levels can be compromised by inherited or acquired defects in the enzymes, transporter, signaling molecules, or transcription factors that are involved in its homeostasis. GSH deficiency accompanies an increased susceptibility to oxidative stress. GSH acts as a proton donor, neutralizes H<sub>2</sub>O<sub>2</sub> and organic peroxides, directly reacts with radical species and contributes to the regeneration of other antioxidants such as tocopherol and ascorbic acid [18].

There is evidence that GSH itself is a neurotransmitter or neuromodulator and any change in either GSH levels, turnover rates, or oxidation state would adversely affect Central Nervous System activity [19-25].

GSH in the serum of autistic patients was found to be significantly lower than in the controls. (Fig.1) Children are more vulnerable than adults to oxidative stress because of the naturally low glutathione levels from conception through infancy [26-27], and the young brain has an immature antioxidant system that may be unable to protect against any increase in ROS. [28]. The risk caused by this natural deficit in detoxification capacity in infants is might be increased by environmental factors that induce oxidative stress [29].

Our results are in agreement with those reported in the literature. Al-Gadani et al. [30] reported the GSH in plasma of Saudi autistic children is significantly lower compared to age-matching controls. Similarly, decreases in the GSH system in brain areas of rats treated with intraventricular propionic acid (PPA) have also been reported [31]. The PPA is an intermediary in cellular fatty acids metabolism and it may play a role in the behavioral, neuropathological and biochemical abnormalities observed in autism. In vitro and animal studies suggest that increased levels of PPA affect diverse processes, including Na<sup>+</sup> K<sup>+</sup> ATPase activity, NMDA glutamatergic receptor activity, cytoskeletal phosphorylation, intracellular calcium levels, scavenging of ROS and modulation of gap junctions [31].

The plasma concentrations of reduced GSH are lower and the ratio of oxidized GSH to reduced GSH is higher in autistic patients compared to healthy controls [32]. Adams et al reported [33] lower levels of ATP, NADH and NADPH in plasma of the autism group than in the reference group and they found a significant correlation with decreased reduced GSH. GSH protects mitochondria against the adverse effects of reactive species and its depletion is associated with impaired mitochondrial function and increased ROS production [34]. Based on this, it is plausible to assume compensatory responses in the antioxidant activity enzymes such as CAT.

Oxidative stress in biological systems usually elicits an antioxidant response; the significant increase in CAT activity level in autistic children could be a compensatory intracellular mechanism to neutralize reactive oxygen species, specifically H<sub>2</sub>O<sub>2</sub>. CAT is involved in recycling the cellular H<sub>2</sub>O<sub>2</sub>; the enzyme is characterized by an elevated reaction capacity and a low substrate affinity, requiring high-level concentration of this substrate to trigger CAT activity [35]. Therefore, some antioxidant enzymes, such as CAT, that are induced by oxidative stress [36], are also widely used as an acceptable oxidative stress marker.

Several studies have suggested alterations in the enzymes that play a vital role in the defense mechanism against damage by ROS in autism. For instance, Zoroglu et al. [9] reported decreased activity of CAT and increased SOD activity in erythrocytes in patients with autism compared to controls. The increase of the SOD activity without the concurrent increase of CAT activity can be the consequence of the oxidative unbalance itself. The excess of H<sub>2</sub>O<sub>2</sub> generated by SOD activity would accumulate ROS and could result in the loss of the catalytic activity of CAT due to post-translational oxidative damage of the protein. Given the role of CAT in the maintenance of oxidant homeostasis, its inhibition or degradation is an exacerbating element of the cellular oxidative damage. In contrast, Sogut et al. [37] reported unchanged plasma SOD activity and increased glutathione peroxidase (GPx). Recently, polymorphisms of GPx 1 were reported to be associated with autism, thus it is possible that the presence of a genetic susceptible variant of GPx and oxidative stress contribute to the autistic phenotype [38].

Our increased CAT enzymatic activity result resembles the increased GPx activity reported by Sogut in plasma from autistic patients [37]. The combined action of CAT and GPx, is indispensable to maintain a low concentration of H<sub>2</sub>O<sub>2</sub>, necessary for an appropriate redox balance. Giulivi et al. [39] reported that the rates of H<sub>2</sub>O<sub>2</sub> production in lymphocytic mitochondria from children with autism were higher than in controls. If H<sub>2</sub>O<sub>2</sub> levels exceed the antioxidant capacity of GPx and CAT, the H<sub>2</sub>O<sub>2</sub> accumulates and it can react with transition metals even in distant places from where it was generated, leading thus to the formation of ·OH, a species able to cause severe damages to the biomolecules, including lipids, proteins and DNA.

Lipid peroxidation is a chain reaction between polyunsaturated fatty acids and ROS, and it produces both lipids peroxides and hydrocarbon polymers, which are highly toxic to the cell. MDA is an end product of the peroxidation of polyunsaturated fatty acids and related esters, and is, therefore, used as a marker of lipid peroxidation. Oxidative stress in autism is associated with increased plasma levels of MDA and others lipid peroxidation biomarkers [40].

The high levels of MDA in the studied autistic patients suggest the existence of an elevated damage to the lipid component of the cell. The MDA concentration correlates directly with ROS – mediated cell damage, specifically ·OH [41]. This result is consistent with the increase of CAT activity and the insufficient concentration of GSH to maintain redox homeostasis in autistic children.

Chauhan et al. [40] reported that the phospholipid composition and the fluidity of the erythrocyte cell membrane are altered in autism. These results suggest that membranes are more rigid in autism. Alteration in membrane lipid metabolism, such as composition of fatty acids, oxidation of lipids and altered levels of phospholipids in the membrane will affect the functions of proteins that are involved in signal transduction such as receptor and receptor-coupled enzymes which are essential for neuronal survival and synaptogenesis [42]. The quaternary structure and function of protein depends on the precise composition of its immediate phospholipid environment [43]. These abnormalities in the membrane lipids may be involved in the etiology of autism.

Likewise, Ming et al. [41] found increased lipid peroxidation associated with autism. Montuschi reported increased excretion of F<sub>2</sub>isoprostane in children with ASD [44]. This isoprostane is a product of nonenzymatic oxidation of arachidonic acid and is widely recognized as reliable marker of lipid peroxidation [44].

DNA damage is an underlying cause of cellular dysfunction and death. Unrepaired DNA can drive mutagenesis that disrupt normal gene expression, or create aberrant proteins in detriment to cellular function or viability. 8OHdG is the product of free radical attack to DNA-bound guanosine. It is the most abundant product of cellular DNA oxidation [45-46] and is also a potent mutagen [47] and hence a marker for oxidative damage to DNA.

Associated with the decrease in GSH content, as antioxidant defense, and the increase in the MDA biomarker, a higher 8OHdG serum level was also found in autistic children compared to healthy controls. (Fig. 1) Sajdel-Sulkowska et al. [48] reported an increase in the 8OHdG in autistic cerebella. A trend toward increased urinary 8OHdG directly in children has been reported in autism [40]. Increased 8OHdG levels have been also observed in lymphocytes [49] and cerebrospinal fluid [50] of patients with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Children with brain damage also show increased urinary 8OHdG levels [51]. Recently, elevated levels of 8-oxo-deoxyguanosine in the cerebellum of patient with autism were reported by Rose et al. [52].

It is well known that autism patients suffer extensive neuronal loss in the cerebellum [53] and recently stereological studies also demonstrated significant neuronal loss in the amygdala area [54]. How these neurons are lost is a matter of debate in the field. Emerging evidence suggests that neurodegeneration may also be involved. Taken together, these results indicate that cumulative oxidative damage over time could influence the onset or severity of autism.

#### **4. CONCLUSION**

In conclusion, the data from this study support the hypothesis of the oxidant/antioxidant balance as a key piece that may contribute to autistic pathology, however, explanation of the underlying molecular mechanism are currently underway. The growing recognition that oxidative stress is involved in the pathogenesis of autism has generated considerable interest to identify early biological markers that may help develop earlier clinical, therapeutic or preventive strategies.

#### **ETHICAL APPROVAL**

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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